AD_____

Award Number: DAMD17-01-1-0199

TITLE: Use of a Transgenic Mouse Model with a Regulatable Estrogen Receptor Alpha (ER) to Study the Role of ER in Mammary Gland Development and Cancer

PRINCIPAL INVESTIGATOR: Sonia L. Sugg, M.D.

CONTRACTING ORGANIZATION: Medical College of Wisconsin
Milwaukee, WI 53226-0509

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (20 Aug 2002 - 19 Aug 2003)

4. TITLE AND SUBTITLE
Use of a Transgenic Mouse Model with a Regulatable Estrogen Receptor Alpha (ER) to Study the Role of ER in Mammary Gland Development and Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0199

6. AUTHOR(S)
Sonia L. Sugg, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical College of Wisconsin
Milwaukee, WI 53226-0509

E-Mail: ssugg@mcw.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Estrogen (E2) is required for the development of breast cancer, but there are few animal models to study its mechanism of action in vivo. We hypothesize that the timing of estrogen receptor α (ERα) action is crucial in normal mammary gland development and tumorigenesis. Therefore, a transgenic mouse model with a regulatable ERα is being generated. In vitro, a mutant ERα (525L) has a severely attenuated response to endogenous estradiol (E2) but a wild-type (WT) response to diethylstibestrol (DES). Gene targeting was used to insert 525L into the WT ER gene. Positive clones were injected into mouse blastocysts and chimeras were generated. There has been no germline transmission so far. Additional injections and further breeding is in progress. The resultant transgenic mouse will enable us to study how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers.

14. SUBJECT TERMS
Breast cancer, estrogen receptor

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Estrogen and its receptor are required for normal breast development and are important in the treatment and prevention of breast cancer. However, there are few suitable animal models to study the role of ER activation in the breast development and cancer. We developed a mutant ER having a severely attenuated response to endogenous estradiol while demonstrating a wild-type response to a synthetic estrogen agonist *in vitro*. This was due to a difference in binding affinity. We postulate that the timing and nature of estrogen action is crucial in normal mammary gland development and tumorigenesis. We are proposing a transgenic mouse model with a regulatable ER to test this hypothesis. Our specific aims are to 1) Develop transgenic mice using gene-targeting techniques expressing only the ligand discriminating mutant ER. 2) Define the contribution of ligand induced transcriptional activation of ER in the development of normal mammary gland. 3) Investigate how the timing of ER activation modulates the development of genetically and chemically induced mammary cancers. The mutant ER mice are uniquely suited to study the role of ligand independent ER activation *in vivo*, and will also be useful in studying reproductive tract development and behavior.

BODY

Task 1. **Transgenic mice expressing only a mutant ER with ligand discrimination will be developed. (months 1 - 12)**

a) Mouse ER genomic DNA will be cloned. A targeting construct (8 to 10 kb) will be generated.
Completed.

b) Mice transgenic and homozygous for mutant ER will be generated.
A two-pronged effort, with 2 different targeting constructs, was started at MCW and the University of Chicago. The rationale was to increase the probability of, and decrease the time required to successfully generating a transgenic mouse.

1) Work performed at the University of Chicago.

An 11.5 kilobase ERα construct containing the G525L mutation (purple bar in green exon 9 box), an 18 bp 6xHis-tag epitope, and an extra Xba1 site, was engineered to facilitate homologous recombination into the mouse genome (Figure 1). The original construct was obtained from Dr. Kenneth Korach[1] and the G525L mutation was inserted in our laboratory by site directed mutagenesis. We also inserted an ACN cassette into the targeting construct, obtained from Dr. Wondisford at the University of Chicago, which contained a testis-specific

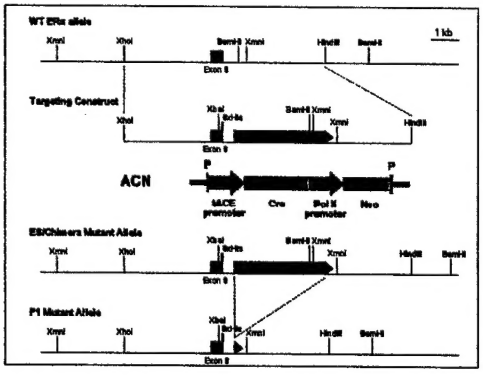

Fig. 1. ERα construct containing the G525L mutation with ACN cassette

promoter (tACE), Cre structural gene, mouse RNA polymerase gene, and neo cassette, flanked at the 5' and 3' ends by loxP sites[2] (Figure 1).

The targeting construct was electroporated into ES cells. Positive clones were identified by 5' external probe Southern blots (Figure 2). A PCR strategy to identify 3' integration was also developed (Figure 3). PCR of exon 9 was used to detect the 6xHis-tag, Xba1 site, and G525L mutation in the positive clones (Figure 4). Southern blots with an internal probe were also performed to demonstrate only one insertion event in each positive clone (Figure 2). Two positive clones, 55 and 71, were obtained. They were injected into mouse blastocysts and four chimeras, one female from clone 55 and two males and one female from clone 71, were generated. Thus far, germline transmission has not been achieved with any of the chimeras.

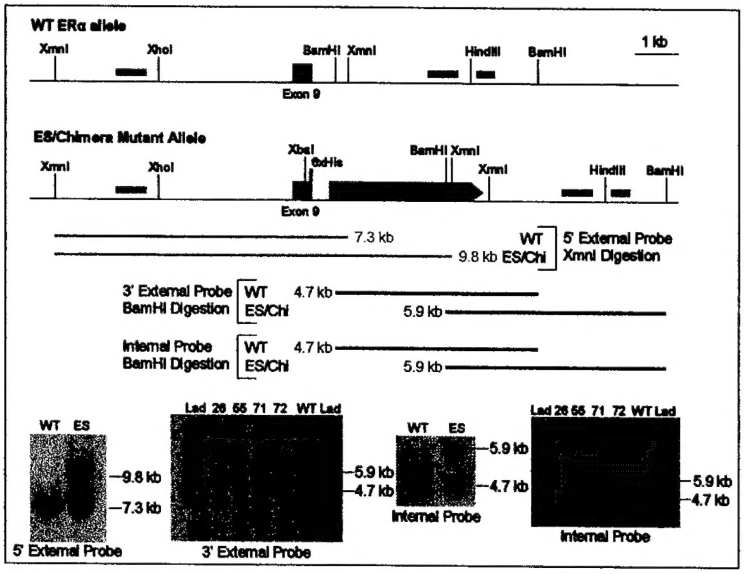

Fig. 2 Identification of positive clones by 5' external probe Southern blots

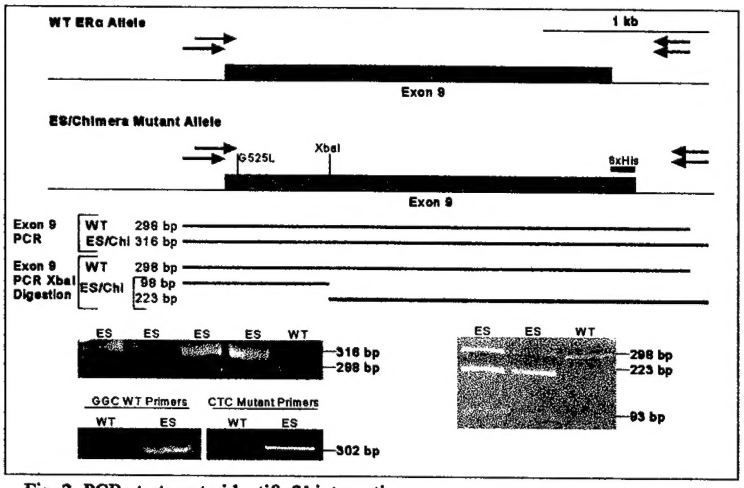

Fig. 3. PCR strategy to identify 3' integration

In the future, electroporation of the targeting construct into fresh ES cells will be repeated. With the assistance of our collaborators, who have extensive expertise in developing knock-in and knock-out mouse models, we are confident that this approach will be successful. If not, an alternate strategy of inserting the mutant ERα into an αERKO background could be used to create a similar model. In this system, ERα expression would be driven by an exogenous promoter.

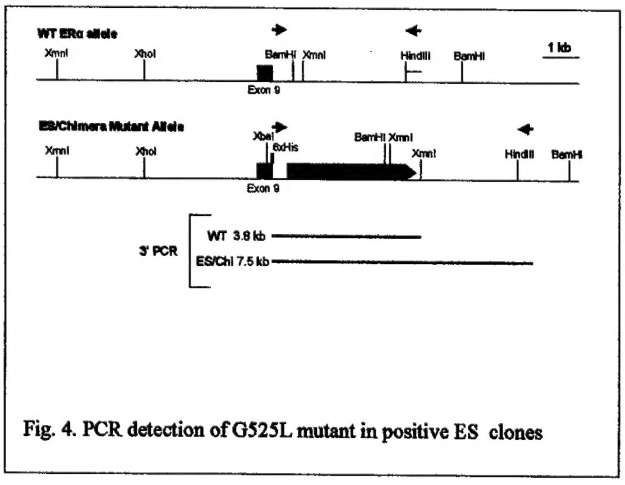

Fig. 4. PCR detection of G525L mutant in positive ES clones

After germline transmission, Southern blot genotyping from tail DNA restriction digest will confirm the excision of the ACN cassette. The tACE promoter will drive expression of the Cre-recombinase gene in the testis of the chimeric mice during spermatogenesis, so that the ACN cassette is self-excised in the ES cell derived sperm of the chimeras[2]. A Southern blot strategy to detect the ACN cassette excision in the F1 generation has been developed (Figure 5).

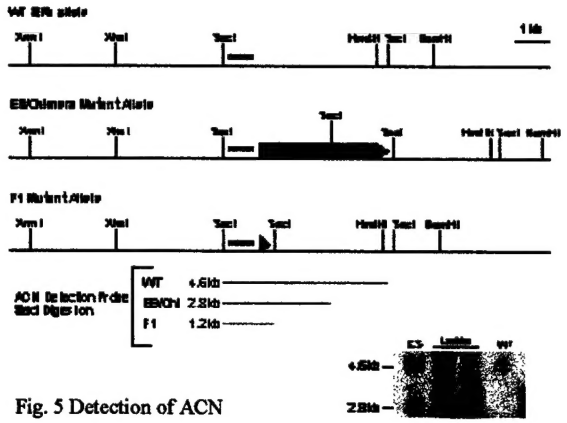

Fig. 5 Detection of ACN

2) Work performed at the Medical College of Wisconsin.

A 15 kb G525L mutant ER targeting construct was used to generate 18/380 (4.7%) positive ES cell clones, as detected by 5' probe Southern blot screening (for details please see last year's report). The positive clones were further confirmed by 3' external probe Southern hybridization[1], PCR of exon 9 followed by restriction enzyme XbaI digest (Figure 4), G525L mutation sequencing, and PCR of the neo selection marker (see Table 1). Three positive clones 29, 200, and 362 were selected for blastocyst injection. The genomic DNA from these 3 clones was again confirmed by all of the above methods to be positive prior to blastocyst injection. Clones 200 and 362 had litters that were aborted or cannibalized. Clone 29 generated 6 chimeras with 60 to 90% coat color chimerism. The first breeding generated no germline transmission. We are continuing to breed clone 29 chimeras and are reinjecting clones 200 and 362.

Table 1

ES Clone#	Southern/5'probe	Seq.PCR/XbaI	Southern/3'probe	Mutation Sequencing	Southern/Neo
#362	++++++		+++	+	+
#29	+++++		+++	+	+
#2	++++		-	N/A	+
#200	++++		+++	+	+
#110	+++		++	+	+
#130	+++		-	+	+
#134	+++	non cut	N/A	N/A	+
#175	+++		-	N/A	+
#241	+++		+	+	+
#244	+++		+++	+	+
#269	+++		-	N/A	+
#299	+++		+++	+	+
#28	++	non cut	N/A	N/A	+
#34	++		-	N/A	+
#225	++	non cut	N/A	N/A	+
#257	++		++	+	+
#331	++	non cut	N/A	N/A	+
#338	++	non cut	N/A	N/A	+
#1	-		-	-	-
#3	-	cut	+++	+	+
#4	-		-	N/A	-
#5	-		-	-	-
#6	-	cut	++	+	-
#13	-		-	N/A	-
#14	-		-	-	-
#16	-		-	N/A?	-
#17	-		++	N/A	+
#18	-		-	N/A	-

KEY RESEARCH ACCOMPLISHMENTS:

1) Generation of mutant ER chimeras from 2 targeting constructs.

REPORTABLE OUTCOMES:

Abstracts:

1) Sugg SL, Li J, Huang JJ, Radek J, Brace J, Swope DL, Korach KS, Greene GL. Design of a transgenic mouse with a regulatable estrogen receptor alpha. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando 2002.
2) Sugg SL, Li J, Radek J, Shilyansky J, Greene GL. Differences in Binding Affinity Causes Mutant Estrogen Receptor Alpha Ligand Discrimination. Proc AACR, 2003.

CONCLUSIONS

Over the second year of this award, we have made substantial progress toward creating the transgenic mouse model with a regulatable ER that was proposed for this project. We anticipate having transgenic animals imminently. We will then be able to characterize the phenotype, followed by experiments to determine how the timing of ER action affects mammary gland maturation and modulates mammary tumorigenesis and latency in models of genetically and chemically induced mammary cancers. With the widespread clinical use of ER ligands, detailed understanding of ER action is increasingly important. This model will allow us to manipulate ER transcriptional activity *in-vivo* during different developmental stages, and will add significantly to current knowledge on ligand-activated and ligand-independent ER action. Results from our proposal on how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers will likely lead to determining susceptible periods for treatment in the prevention of breast cancer in humans.

REFERENCES

1. Swope DL, Castranio T, Harrell JC, Mishina Y, Korach KS. AF-2 knock-in mutation of estrogen receptor α: cre-loxP excision of a PGK-neo cassette from the 3' UTR. Genesis. 2002;32:99-101.

2. Bunting M, Bernstein KE, Greer JM, Capecchi MR, Thomas KR. Targeting genes for self-excision in the germ line. Genes & Development. 1999;13:1524-8.